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Research Paper

Human Circulating MicroRNA-1 and MicroRNA-126 as Potential Novel Indicators for Acute Myocardial Infarction

Guangwen Long*, Feng Wang*, Quanlu Duan, Fuqiong Chen, Shenglan Yang, Wei Gong, Yan Wang, Chen Chen✉ and Dao Wen Wang✉

Department of Internal Medicine and the Institute of Hypertension, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, People's Republic of China.

* contributed equally to this study.

✉ Corresponding author: Dao Wen Wang, MD, PhD or Chen Chen, MD, PhD. Department of Internal Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095# Jiefang Ave., Wuhan, 430030, P. R. China. Tel and Fax: (86-27)8366-3280. E-mail: dwwang@tjh.tjmu.edu.cn or chenchen@tjh.tjmu.edu.cn

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Abstract

Circulating miRNAs have been shown as promising biomarkers for various pathologic conditions. The aim of this study was to clarify that circulating miR-1 and miR-126 in human plasma might be useful as biomarkers in acute myocardial infarction (AMI). In our study, after pre-test, two candidate miRNAs were detected by using real-time RT-PCR. Cardiac troponin I (cTnI) concentrations were measured by ELISA assay in plasma from patients with AMI (n=17) and healthy subjects (n=25), simultaneously. Increased miR-1 and decreased miR-126 in plasma from patients with AMI after the onset of symptoms compared with healthy subjects were found. A remarkable finding in this study is that miR-1, miR-126 and cTnI expression levels exhibited the same trend. Our results suggest that the plasma concentrations of miR-1 and miR-126 may be useful indicators for AMI.

Key words: circulating miRNA; biomarker; AMI.

Introduction

MicroRNAs (miRNAs) are a relatively novel class of endogenous, non-protein coding small RNA molecules that can regulate hundreds of genes through binding to the 3′ UTR of target mRNAs [1-3]. MiRNAs have been proven to play important roles in self-renewal, cellular development, differentiation, proliferation and apoptosis [4, 5]. Mature miRNAs bind to specific regions of mRNA targets by a mechanism that is not completely understood, resulting in translational repression or mRNA degradation [6-9].

The human genome encodes more than 1,000 miRNAs with tissue or cell type specific expressions [10-13]. Studies have shown that miRNAs have been observed in a wide range of human diseases, including autoimmune, inflammatory, neurodegenerative and cardiovascular diseases [14, 15]. There are mounting evidences showing that cardiac-specific miRNAs, miR-1, miR-133a/b and miR-208, are involved in heart development and cardiovascular diseases, including acute myocardial infarction (AMI) in experimental animals [16]. Recent studies have shown that some miRNAs are present in the systemic circulation, both in humans and animals, and are associated with exosomes and microparticles [17-20]. The levels of some circulating miRNAs have been reported to be differentially expressed during pregnancy and in the presence of a variety of cancers or cardiovascular diseases.

High mortality and morbidity is common in AMI. Some biomarkers have been applied in diagnosis of AMI; for example, cardiac troponin I (cTnI), MB and so on. New approaches that can improve current diagnosis for AMI are still lacking.

However, the expression and possible roles of miRNAs in AMI are less well studied. Cardiac injury, which occurs after AMI increases the circulating levels of several myocardial-derived miRs (eg, miR-1, miR-133, miR-499, miR-208), whereas patients with coronary artery disease or diabetes showed reduced levels of endothelial-enriched miRs, such as miR-126 [21]. In the present study, we assessed the hypothesis that miR-1 and miR-126 might be useful for identifying and evaluating AMI. Our aim was to look for miRNA-based modulation of gene expression in AMI.

Materials and Methods

Blood Samples

This study was supported by the Ethics Committee of Tongji Hospital, and was performed in accordance with the principles of the Declaration of Helsinki. After gaining their written informed consents, we gathered blood samples from 17 patients and 25 healthy adults at Tongji Hospital between October 2009 and May 2010. AMI was diagnosed based on combination of several criteria: 1) ischemic symptoms; 2) creatine kinase-MB (CK-MB); 3) pathological Q wave; 4) increased cTnI; and 5) ST-segment elevation or depression [22]. In addition, 25 healthy adult volunteers (normal electrocardiogram and no history of cardiovascular diseases) were enrolled in this study. The blood samples of patients with AMI were acquired at 4h (± 30min), 8h (± 30min), 12h (± 30min), 24h (± 30min), 48h (± 30min), 72h (± 30min) and 1w (± 60min) after the onset of symptoms. Plasma was isolated by centrifugation and conserved at -80°C until purification.

RNA Purification

Total RNA was extracted from plasma with TRIzol LS Reagent as described previously [23].

Plasma cardiac troponin I determine

Plasma cTnI concentrations were measured by ELISA assay according to the manufacturer's protocol (Abnova, Taiwan).

MiRNA qRT-PCR

Two μg of total RNA were reverse-transcribed using Transcript First-strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) in accordance to the manufacturer's protocol. In short, the 50 uL reactions were incubated for 60 min at 42°C, 10 min at 70°C, and then stored at 4°C.

qRT-PCR were analyzed using the Bulge-Loop™ miRNA qRT-PCR Detection Kit (Ribobio Co., Guangzhou, China) and TransStart™ Green qPCR SuperMix (TransGen Biotech, Beijing, China) in accordance with the manufacturer's protocol with the Rotor-Gene 6000 system (Corbett Life Science, QIAGEN, Hilden, Germany). Briefly, the reactions were incubated at 95°C for 30s, followed by 40 cycles of 95°C for 30s, 60°C for 20s, 70°C for 1s. The relative expression level for each miRNA was computed using the comparative CT method. It is important to note that to control for possible diversity in the amount of starting RNA, miRNA expression was normalized to small nucleolar RNA U6.

Statistical analysis

A widely used method to present relative expression of miRNA is the $2^{-\Delta\Delta ct}$ method. Relative expression of miRNA presents the data of the miRNA of interest ($Ct_{miRNA\ of\ interest}$) relative to internal control gene ($Ct_{internal\ control\ miRNA}$), termed Δct. Results are calculated as Δct± standard deviation (SD). Different groups of AMI patients and healthy adults were compared with Δct±SD of the control group (healthy adults) and tested for statistical significance. When the differences in Δct±SD of the tested groups reached or were below $p = 0.05$, the difference in miRNA expression was considered statistically significant. The data were presented as fold change graphically. Fold change of the tested group is calculated relative to the control group (healthy adults) using the $2^{-\Delta\Delta ct}$ equation.

All values of miRNAs are expressed as mean±SD. Comparisons of parameters among ≥ 3 groups were analyzed by repeated-measures ANOVA. Independent-sample T test was used for 2-group comparisons. For categorical variables, Chi-Square test was used. Time-course trends of miRNAs and cTnI were analyzed by repeated-measures ANOVA. Differences were considered statistically significant at a value of $P<0.05$.

A score (miRNA-score) was defined as the level of miR-1 and miR-126 in the AMI group as compared with the control group. The miR-1 and miR-126-score of each sample was calculated as the sum of the inverted-normalized signals of the miR-1 and miR-126-scores and adjusted by subtracting a constant (the minimal score), so that the range of scores starts at 0 [24].

For all statistical analyses, the statistical software SPSS 13.0 for Windows was used.

Results

Statistical analysis of patients' characteristics

Among 17 patients with AMI, 13 were males and 4 females; their age ranged between 40 and 70 years (mean 53±12.5). All patients had a transmural MI. There were no statistical differences between the control group and the AMI patients for any of the considered variables such as LDL cholesterol, total cholesterol, total triglycerides, HDL cholesterol, white blood cell count, systolic blood pressure, diastolic blood pressure, creatinine and history of diabetes and smoking status were recorded respectively. Details were shown in Table 1.

Table 1. Clinical characteristics of patients.

Characteristics	Total patients (n=42)	AMI (n=17)	Healthy adults (n=25)	p
Age (years)	52.5±11	53±12.5	51±12.3	0.42
Male/female (n/n)	31/11	13/4	18/7	0.75
WBC (*10^9/L)	7.3±1.8.0	8.2.0±2.4	6.1±1.35	0.083
HDL C (mmol/L)	1.14±0.25	1.08±0.23	1.23±0.28	0.56
TG (mmol/L)	1.52 ±1.2	1.36±0.73	1.48±1.37	0.76
Hyperlipidaemia, n (%)	4 (10%)	2 (12%)	2 (8%)	0.35
Cr (umol/L)	66±30.5	85±51	54±15	0.46
SBP (mmHg)	126. ±14	138±22	120 ±16	0.82
DBP (mmHg)	82 ±14	84±16	76±12	0.46
Current smoking, n (%)	23 (63 %)	12 (75%)	11 (55%)	0.089
Fasting glucose (mmol/L)	5.46±1.53	6.07±1.23	5.23±1.63	0.5
TC (mmol/L)	4.32±0.65	4.02±0.	4.15±0.650.82	0.8
LDL C (mmol/L)	2.27±0.57	2.58±0.6	2.23±0.54	0.6
DM, n (%)	3 (7.%)	2 (12%)	1 (4%)	0.36
Hypertension, n (%)	16 (38 %)	8 (50%)	8 (30%)	0.324

DM, diabetes mellitus; TC, total cholesterol; HDL C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL C, low-density lipoprotein cholesterol; WBC, white blood cell; Cr, creatinine. P, comparison between patients with healthy adults. TG, total triglyceride.

MicroRNAs plasma levels in AMI and healthy adult

Using qRT-PCR, we analyzed the expression of two miRNAs in AMI patients and in healthy adults. The results were summarized in Table 2 and Figure 1. The data showed that plasma levels of miR-1 exhibited 3.63 (± 0.94) fold, 15.87 (± 4.5) fold, 5.9 (± 1.03) fold, 2.37 (± 0.50) fold, 4.9 (± 1.34) fold, 4.3 (± 1.2) fold and 2.38 (± 0.58) fold increase at 4h (± 30min), 8h (± 30min), 12h (± 30min), 24h (± 30min), 48h (± 30min), 72h (± 30min) and 1w (± 60min), respectively (Table 2 and Fig. 1A). miR-126 levels in AMI patients were 78%, 48%, 64%, 90%, 58%, 82% and 93% lower than in healthy controls at 4h (± 30min), 8h (± 30min), 12h (± 30min), 24h (± 30min), 48h (± 30min), 72h (± 30min) and 1w (± 60min) (Table 2 and Fig. 1B), respectively. Then, circulating miR-1 and miR-126 expression time courses were analyzed by repeated-measures ANOVA. As shown in Fig. 1, miR-1 and miR-126 plasma levels in AMI at each time point were compared. MiR-1 expression was obviously up-regulated at 8h (± 30min) compared with other time points (Fig. 1A), while miR-126 concentration was clearly down-regulated at 24h (± 30min) and 1w (± 60min) compared with other time points (Fig. 1B).

Simultaneous microRNAs and cTnI plasma levels determination in AMI patients

Plasma cTnI concentrations were measured by ELISA assay according to the manufacturer's protocol. In 17 patients, miRNAs and cTnI were measured from the same plasma samples. Compared with healthy adults, cTnI peak increase of 2584 (± 646) fold was achieved at 8h. cTnI increased 2555 (± 655) fold, 1748 (± 437) fold, 1919 (± 329) fold, 892 (± 244) fold, 611 (± 44) fold and 11.3 (± 1.3) fold at 4h, 12h, 24h, 48h, 72h and 1w, respectively (Fig. 1C and 1D). Meanwhile, miR-1, miR-126 and cTnI time courses exhibited the same trends in these patients, by repeated-measures ANOVA analysis. Importantly, no significant interaction effects were found in the time course between miRNAs and cTnI (Fig. 1C and 1D).

Table 2. MiR-126 and miR-1 in human AMI groups and healthy adults.

		MI (4h)	MI (8h)	MI (12h)	MI (24h)	MI (48h)	MI (72h)	MI (1w)	healthy adult
miR-126	(Δct±SD)	2.61±0.5	1.35±0.33	1.85±0.27	3.66±0.82	1.64±0.34	2.89±0.59	4.23±0.38	0.40±0.12
p value		0.01	0.02	0.04	0.01	0.02	0.01	0.01	
AUC		0.86	0.87	0.88	0.87	0.84	0.83	0.78	
miR-1	(Δct±SD)	8.14±2.11	6.0±1.72	7.4±1.3	8.76±1.83	7.7 ±2.1	7.9 ±2.2	8.76±2.15	10.0±2.12
p value		0.001	0.02	0.04	0.03	0.003	0.01	0.02	
AUC		0.92	0.90	0.94	0.92	0.96	0.90	0.76	

Δct value of miR-126 and miR-1 in human AMI groups and healthy adults is presented as an average group Δct±SD. Corresponding p values were calculated using independent-samples T test, and missing p values represent non significant Δct changes. AUC indicates the area under the receiver operating characteristic (ROC) curve for the differentiation between AMI and control groups.

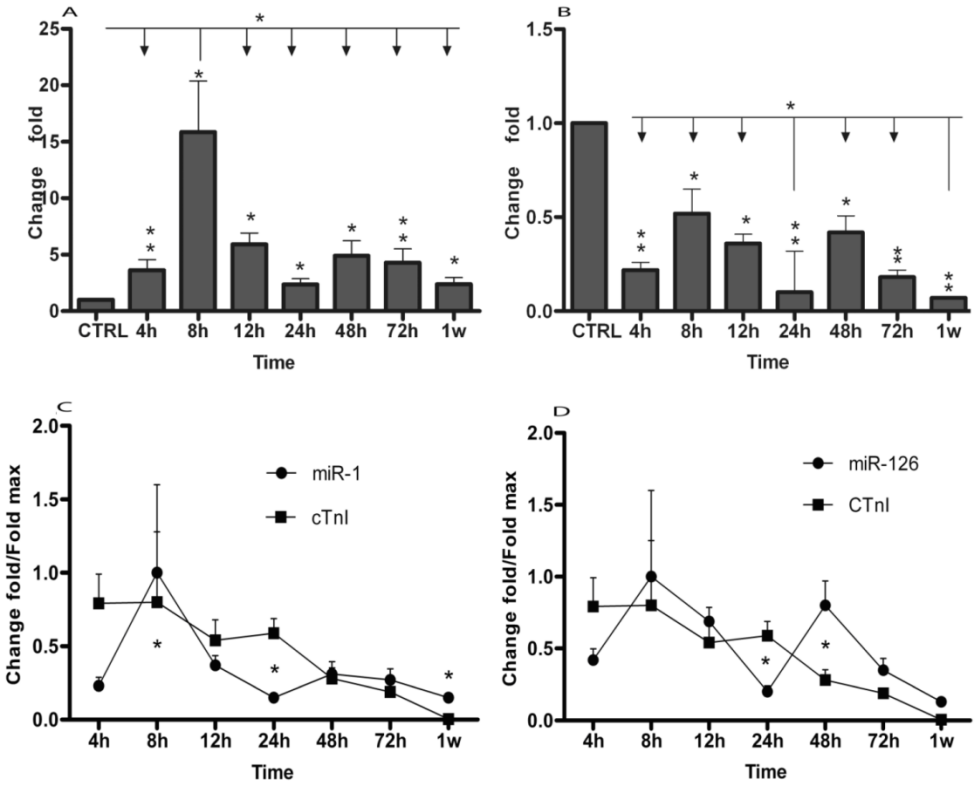

Figure 1. MicroRNAs expression of plasma in patients with AMI. MicroRNAs plasma levels in patients with AMI using real-time PCR assays. Plasma samples were collected at 4h (± 30min), 8h (± 30min), 12h (± 30min), 24h (± 30min), 48h (± 30min), 72h (± 30min) and 1w (± 60min) after the onset of symptoms. (A) Expression level of miR-1 at different time points; (B) Expression level of miR-126 at different time points; Time courses of circulating miRNA and cTnI from the same plasma samples in patients with AMI and healthy control subjects; (C) Circulating miR-1 expression at different time points; (D) Circulating miR-126 expression in different time points; Values show fold changes of each microRNA vs. its level in the control group, arbitrarily set at 1 as indicated by CTRL, and fold changes of cTnI vs. its level in control group, arbitrarily set at 0.8 as indicated by the CTRL. The data were normalized to the peak level for each microRNA and cTnI achieved in each patient and the time of the peak-fold increase vs. results are reported as mean±SD (*, $p< 0.05$; **, $p\leq 0.01$).

Specificity and sensitivity of miR-1 and miR-126

Since the detected levels of miR-1 and miR-126 in plasma may be affected by both technical and biological variation, we combined the levels of miR-1 and miR-126 into a single score to provide an improved signal to noise ratio. The miR-1-score and miR-126-score stand for the plasma levels of the miR-1 and miR-126 with $P<0.0001$ for the comparison between AMI and control group, was calculated as described in the methods section. The miR-1 and miR-126-scores allowed a significant separation between the AMI and control groups ($P = 1e-7$). The median score of miR-1 is 2.16, 2.55, 2.85, 2.58, 2.52 and 2.10 in the AMI group. It is 1.04, 1.39, 1.25, 1.1, 0.96 and 1.08, respectively, in the control group at 4h, 8h, 12h, 24h, 48h and 72h (Figure 2). The ability of the miR-1-score to differentiate the AMI group from the control group is demonstrated by the ROC curve with an AUC of 0.92, 0.90, 0.94, 0.92, 0.96 and 0.90 (Figure 3 and Table 2). By using a threshold score of 1.57, 2.03, 2.26, 1.47, 1.83 and 1.74 above which patients are predicted to belong to the AMI group, we achieved a sensitivity of 93%, 93%, 94%, 93%, 93% and 90% and a specificity of 90%, 90%, 93%, 90%, 90% and 90%, respectively, for the identification of AMI patients. The median score of miR-126 is 2.51, 2.55, 2.5 2.6, 2.4 and 2.2 in the AMI group. It is 1.31, 1.31, 1.30, 1.35, 1.21 and 1.20 in the control group at 4h, 8h, 12h, 24h, 48h and 72h (Figure 4). The ROC curve of miR-126 showed moderate ability to distinguish between the AMI group and the healthy control group at 4h, 8h, 12h, 24h, 48h and 72h with an AUC of 0.86, 0.87, 0.88, 0.87, 0.84 and 0.83 (Figure 5 and Table 2), respectively. Using a threshold score of 1.6, 1.59, 1.88, 1.7, 1.2 and 1.25 in the AMI group, we gained a specificity of 78%, 84%, 88%, 77%, 80% and 70% and a sensitivity of 81%, 81%, 81%, 81%, 81% and 81%, respectively, for the diagnosis of AMI in patients.

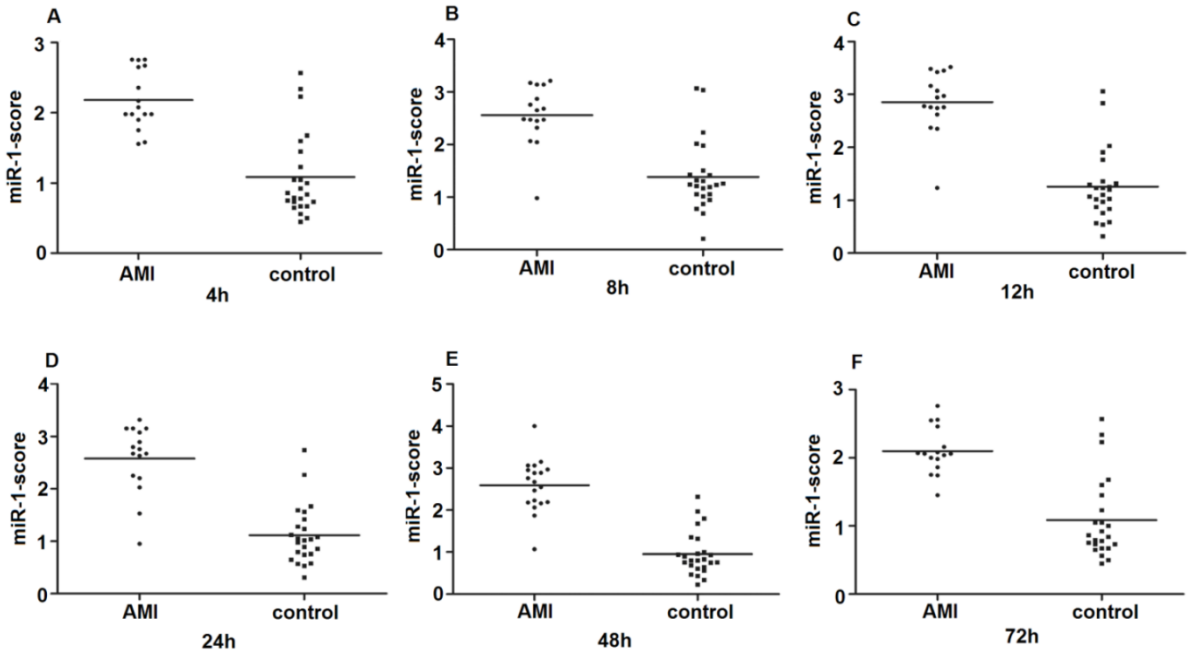

Figure 2. Plasma miR-1-score in AMI groups and control group. Differences between various time points after the onset of AMI and control groups using the miR-1-score are presented. (A) 4h; (B) 8h; (C) 12h; (D) 24h; (E) 48h; (F) 72h.

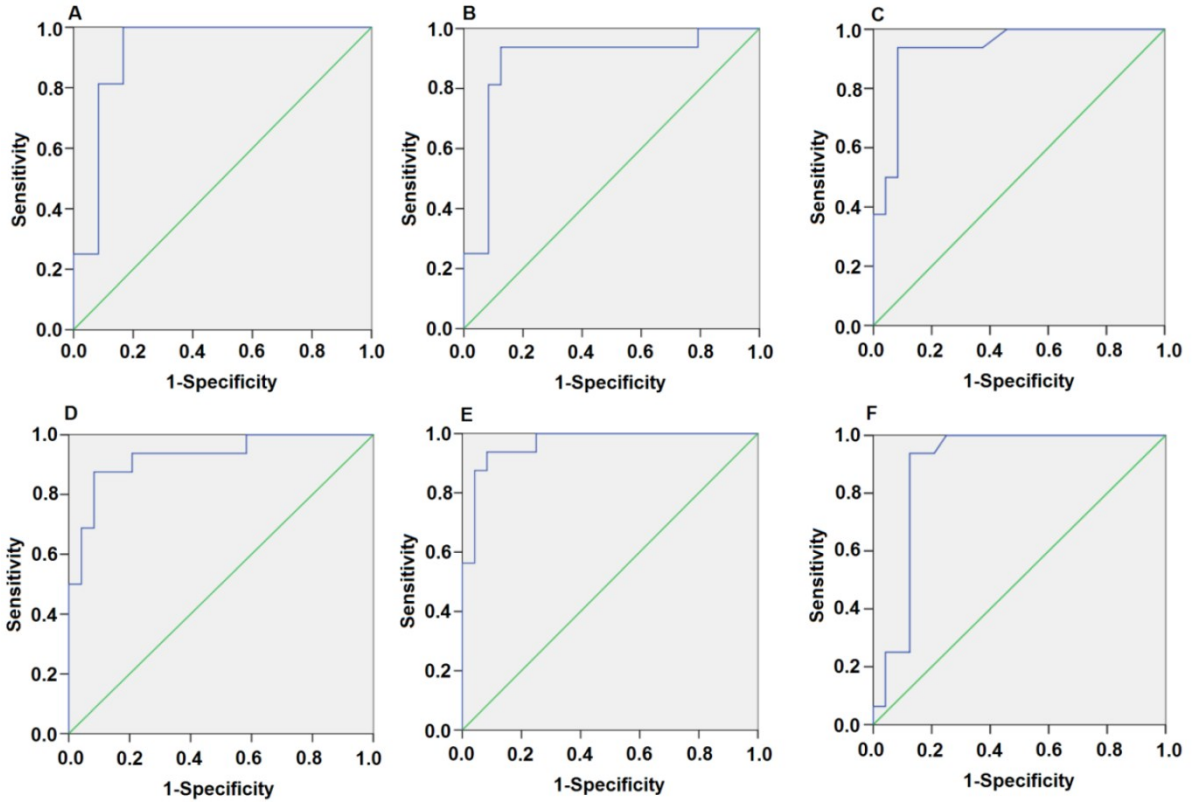

Figure 3. Plasma miR-1 is significantly associated with AMI. Receiver operating characteristic (ROC) curve for miR-1-score discriminates between various time points after the onset of AMI and control groups using the miR-1-score. (A) 4h; (B) 8h; (C) 12h; (D) 24h; (E) 48h; (F) 72h.

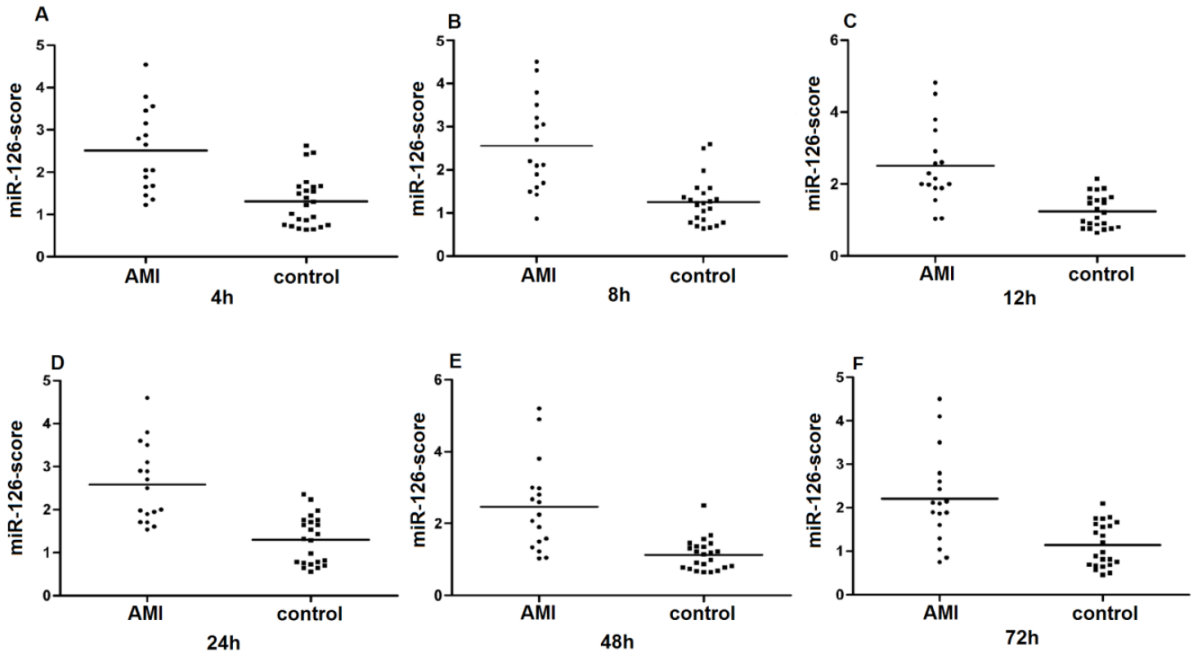

Figure 4. Plasma miR-126-score in AMI groups and control group. Plasma miR-126-score is shown between AMI groups and control group at 4h, 8h, 12h, 24h, 48h and 72h. Lines show median values, which were 2.51, 2.55, 2.5, 2.6, 2.4 and 2.2 in the AMI group and 1.31, 1.31, 1.3, 1.35, 1.21 and 1.2 in the control group (P = 1e-7) (A, B, C, D, E and F).

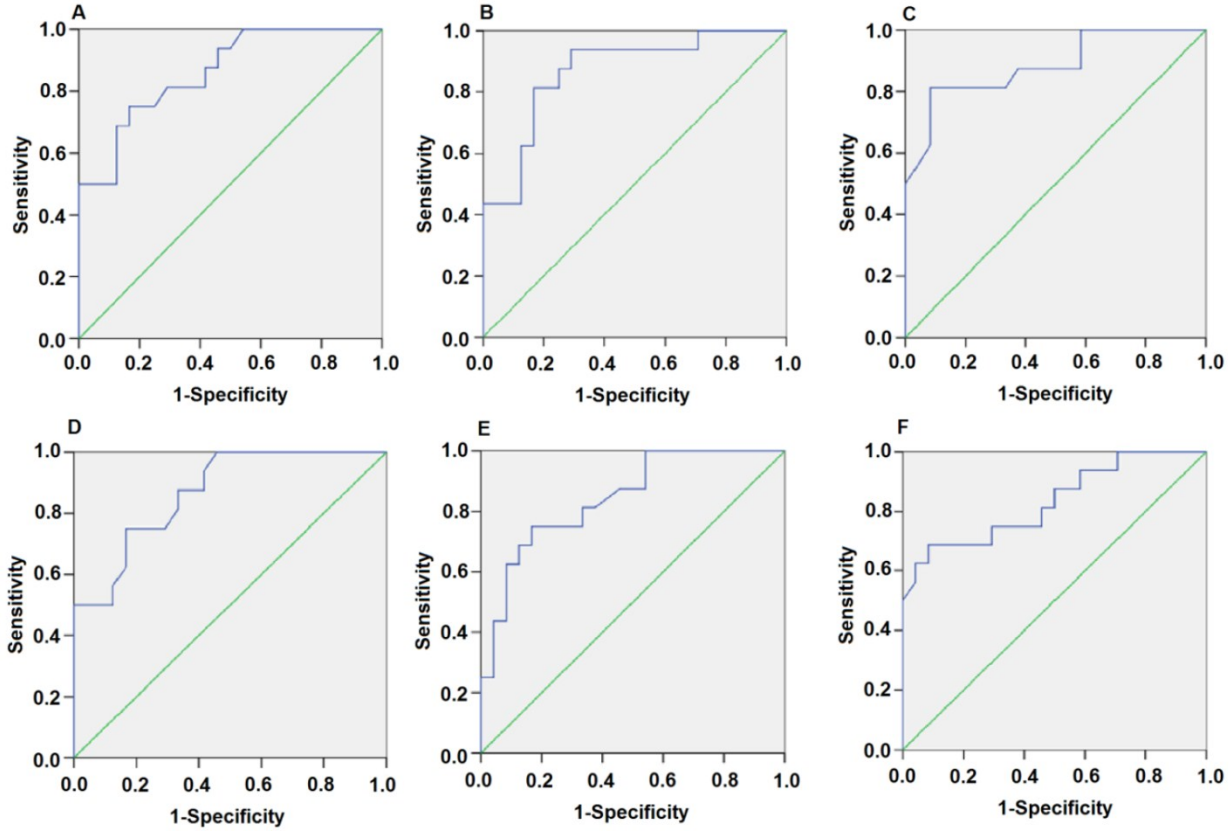

Figure 5. Plasma miR-126 is strongly associated with AMI. Receiver operating characteristic (ROC) curve analysis of the diagnostic value of circulating miR-126 for AMI at 4h, 8h, 12h, 24h, 48h and 72h. The areas under the curve (AUC) are 0.86, 0.87, 0.88, 0.87, 0.84 and 0.83 (A, B, C, D, E and F).

Discussion

MiRNA regulates gene expression by binding and modulating the translation of specific miRNAs [25-28]. In recent years, tremendous efforts have been made to use a miRNA microarray screening approach to address the miRNA expression profiling in infarted hearts from animal models [28-30]. Recent evidences suggested that miRNAs are excellent promising disease biomarkers [31-33]. MiRNAs have been consistently detected in serum, plasma and other bodily fluids and specific miRNA patterns have been associated with pregnancy and with certain diseases [34-36]. MiRNAs have been shown to play important roles in development, stress responses, angiogenesis and oncogenesis. Accumulating evidence also points to important roles of miRNAs in the cardiovascular system [8, 37]. However, the role of miRNA in AMI is just emerging.

In this study, we reported the circulating expressions of miR-1 and miR-126 in AMI patients, in comparison to healthy adults. The most significant finding was that plasma levels of miR-126 were down-regulated in acute myocardial infarction at 4h (± 30min), 8h (± 30min), 12h (± 30min), 24h (± 30min), 48h (± 30min), 72h (± 30min) and 1w (± 60min) after the onset of AMI symptoms. Importantly, miR-1 was up-regulated at 4h (± 30min), 8h (± 30min), 12h (± 30min), 24h (± 30min), 48h (± 30min), 72h (± 30min) and 1w (± 60min) after the onset of AMI symptoms. A remarkable finding in this study is that miR-1, miR-126 and cTnI exhibited the same trends, however, with no significant interaction effects. The plasma concentration of miR-1 and miR-126 showed a good correlation with the plasma concentration of cTnI, a classic biomarker of myocardial injury. The validity of circulating cTnI as a marker of myocardial injury has been reported [36]. In our study, the plasma concentrations of cTnI and miR-1, miR-126 were highly correlated and exhibited similar time courses. Using the levels of the miR-1 and miR-126, we were able to define a score with a high sensitivity and specificity for the detection of AMI patients at 4h, 8h, 12h, 24h and 72h relative to control group. Furthermore, to look into the possible mechanisms of miR-1/miR-126 in AMI, computational predictions as described at MICROCOSM, miRanda, TargetScan and Pic Tar were employed. These analyses yielded 901/937, 7204/815, 790/25 and 535/21 potential candidates. Some of them were reported to be associated with coronary diseases, such as GJA1, KCNJ2 [38], tanshinone IIA [39], LRP6 [40]. Taken together, our results clearly support that miR-1 and miR-126 may be useful potential biomarker of AMI.

To avoid possible confounders derived from patient selection, age, gender, total triglyceride, creatinine, HDL cholesterol, LDL cholesterol, white blood cell count, total cholesterol, systolic blood pressure, diastolic blood pressure, history of diabetes and smoking status, all these were recorded in our study. Statistical analyses showed that they have no influence on plasma miRNA levels. This suggested that miR-1 and miR-126 were potential biomarkers for certain diseases.

The present study confirmed that miR-1 and miR-126 can be used as biomarkers of AMI in humans. The next question is whether assessment of plasma miR-1 and miR-126 concentration has any clinical significance. MiRNAs might be an especially rich source of diagnostic, prognostic and predictive information as biomarkers in AMI patients [41].

It should be noted that the present study of circulating miR-1 and miR-126 as potential biomarkers for AMI was conducted in a relatively small sample size and future evaluation in larger clinical studies will be needed to confirm the utility as a biomarker. Nonetheless, our study provides a solid basis to identify and develop miR-1 and miR-126 as a novel class of blood-based biomarkers for AMI in the future.

In summary, we found that circulating miR-1 and miR-126 of patients with AMI were significantly changed in a time dependent manner. This suggests that miR-1 and miR-126 have potential utility as novel potential biomarkers for clinic diagnosis of AMI.

Acknowledgments

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Conflict of Interests

None.

Abbreviations

miRNAs: microRNAs; AMI: acute myocardial infarction; cTnI: cardiac troponin I.

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